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Preparation of giant unilamellar vesicles from damp lipid film for better lipid compositional uniformity

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ABSTRACT

Giant unilamellar vesicles (GUVs) containing cholesterol often have a wide distribution in lipid composition. In this study, GUVs of 1,2-dioleoyl-sn-glycero-3-phosphocholine(DOPC)/1,2-distearoyl-sn-glycero-3-phosphocholine(DSPC)/cholesterol and 1,2-diphytanoyl-sn-glycero-3-phosphocholine(diPhyPC)/1,2-dipalmitoyl-sn-glycero-3-phosphocholine(DPPC)/cholesterol were prepared from dry lipid films using the standard electroformation method as well as a modified method from damp lipid films, which are made from compositional uniform liposomes prepared using the Rapid Solvent Exchange (RSE) method. We quantified the lipid compositional distributions of GUV by measuring the miscibility transition temperature of GUVs using fluorescence microscopy, since a narrower distribution in the transition temperature should correspond to a more uniform distribution in GUV lipid composition. Cholesterol molecules can demix from other lipids in dry state and form cholesterol crystals. Using optical microscopy, micron-sized crystals were observed in some dry lipid films. Thus, a major cause of GUV lipid compositional heterogeneity is the demixing of lipids in the dry film state. By avoiding the dry film state, GUVs prepared from damp lipid films have a better uniformity in lipid composition, and the standard deviations of miscibility transition temperature are about 2.5 times smaller than that of GUVs prepared from dry lipid films. Comparing the two ternary systems, diPhyPC/DPPC/cholesterol GUVs has a larger cholesterol compositional heterogeneity, which directly correlates with the low maximum solubility of cholesterol in diPhyPC lipid bilayers (40.2 ± 0.5 mol%) measured by light scattering. Our data indicate that cholesterol interacts far less favorably with diPhyPC than it does with other PCs. The damp lipid film method also has a potential of preparing GUVs from cell membranes containing native proteins without going through a dry state.

1. Introduction

Understanding the organization and dynamics of lipid bilayers is important to the understanding of processes taking place in cell membranes. Since giant unilamellar vesicles (GUVs) were produced for the first time, they have become an indispensable tool for membrane biophysics research. GUVs are cell-sized model membrane systems that allow direct visualization of membrane-related phenomena using optical microscopy. GUVs have been widely used for various investigations, including mapping phase diagrams [1,2], investigating protein–lipid interactions [3–5], investigating 2D phase transitions [1,6,7] and determining line tension between lipid domains [8–10]. Previous studies showed that micron-scale domains in GUVs can be observed in some ternary lipid mixtures composed of a high melting temperature lipid, a low melting temperature lipid and cholesterol [10–12]. Fluorescent dyes can partition preferentially into different lipid phases in GUVs, which allows visualization of these phases using fluorescence microscopy [13].

The electroformation method has become the most widely used GUV preparation method ever since it was developed by Angelova and Dimitrov [14]. Various modifications have been made to the original method to expand its capabilities. For example, GUVs can be made in ionic solution [15–17] and charged lipids can also be included [18]. One important issue about GUVs is the large variation in transition temperature and lipid composition within the same preparation [1,7,9]. Since membrane properties are often sensitive to lipid composition, this drawback can cause large uncertainties in transition temperature measurement and phase diagram determination.

In this study, we investigate lipid compositional heterogeneity in DOPC/DSPC/cholesterol and diPhyPC/DPPC/cholesterol ternary mixtures, which have been extensively studied by other groups [2,7,9]. The chemical structures of these lipid molecules are graphed in Fig. 1. We quantified the lipid compositional distributions of GUV by measuring the miscibility transition temperature of GUVs using fluorescence microscopy, since a narrower distribution in the transition temperature should correspond to a more uniform distribution in GUV lipid composition. We found that the distributions of transition temperature are wide and a major cause of lipid compositional heterogeneity is the demixing of lipids in the dry film state. Comparing the two ternary systems, diPhyPC/DPPC/cholesterol GUVs have

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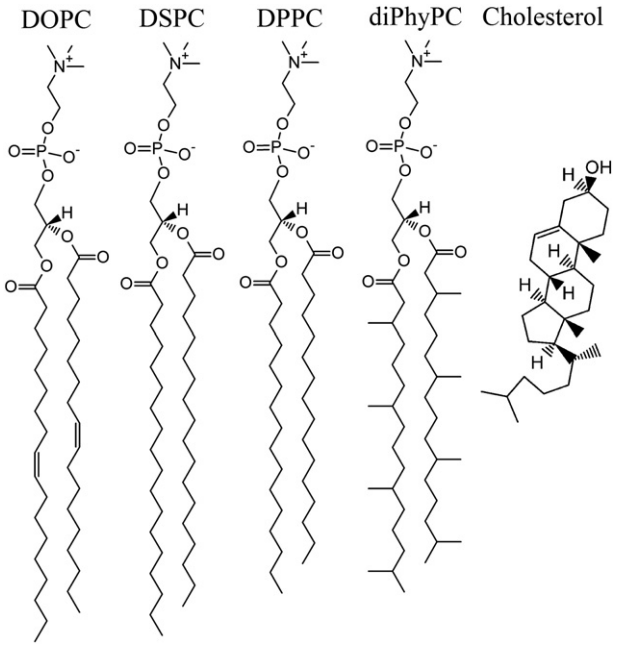

Fig. 1. Chemical structures of DOPC, DSPC, DPPC, diPhyPC and cholesterol.

far larger cholesterol compositional heterogeneity. Using optical microscopy, micron-sized cholesterol crystals were observed in some dry lipid films. Our light scattering experiment showed that cholesterol has a low maximum solubility in diPhyPC lipid bilayers (40.2 ± 0.5 mol%), which indicates that cholesterol interacts far less favorably with diPhyPC than it does with other PCs. This unfavorable interaction between cholesterol and diPhyPC worsens the demixing of lipids in dry film state and results in a large compositional heterogeneity for diPhyPC/DPPC/cholesterol GUVs.

In order to reduce the lipid compositional heterogeneity, we developed a modified procedure that forms GUVs from damp lipid films, which are made from compositionally uniform liposomes prepared using the Rapid Solvent Exchange (RSE) method [19,20]. The new procedure avoids the dry film state, and GUVs prepared from damp lipid films have more uniform lipid composition. Our data shows that the standard deviations of miscibility transition temperature are about 2.5 times smaller than those of GUVs prepared from dry lipid films. The damp lipid film method also has the potential to form GUVs from cell membranes containing native proteins without going through a dry state.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-diphytanoyl-sn-glycero-3-phosphocholine (diPhyPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhodamine-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). 1,1′-Didodecyl-3,3,3′,3′-Tetramethylindocarbocyanine Perchlorate ($DiIC_{12}(3)$) was purchased from Invitrogen (Carlsbad, CA). Cholesterol was purchased from Nu Chek Prep, Inc. (Elysian, MN). Rhodamine-PE and $DiIC_{12}(3)$ are fluorescence probes which preferentially partition into the liquid-disordered (l_d) lipid domains. Purity of the phospholipids (>99%) was confirmed by thin layer chromatography (TLC) on washed, activated silica gel plates (Alltech Associates, Deerfield, IL) and developed with a 65:25:4 chloroform/methanol/water mixture. The molarities of the phospholipid stocks were determined by phosphate assay [21]. Indium Tin Oxide (ITO) coated slides were purchased from Delta Technologies (Loveland, Colorado).

2.2. Preparation of GUV by electroformation from dry lipid film

The lipid compositions of ternary mixtures were selected according to the phase diagrams of DOPC/DSPC/cholesterol [2] and diPhyPC/DPPC/cholesterol [7] to ensure that both mixtures contain coexisting liquid-ordered and liquid-disordered (l_o–l_d) lipid domains at room temperature. The 1:1:1 DOPC/DSPC/cholesterol mixture was labeled with 1% $DiIC_{12}(3)$, and the 3:3:4 diPhyPC/DPPC/cholesterol mixture was labeled with 1% Rhodamine-PE. Our method of GUV preparation from dry lipid films is similar to that used by other groups [2]. Lipid mixtures were first dissolved either in chloroform or 2:1 chloroform/methanol solvent to reach a concentration of 8–10 mM. 10 μl of lipid mixture were then spread on the conducting surfaces of two ITO coated glass slides. The slides were then kept in a vacuum chamber for about 5 h to remove solvent residue. The final pressure of the vacuum chamber was around 20 mTorr. The ITO coated slides were placed with their conducting sides facing each other and separated by Buna o-rings which form a chamber over the dry lipid films, and 400 μl of 150 mM sucrose solution was added to the chamber. Two slides were held together by a small metal clip and electric connections were made through two mini-alligator clips. The sample was then placed on a dry heating block and a 10 Hz sinusoidal voltage of amplitude of 1 V was applied through a function generator for 3 h. Temperature for DOPC/DSPC/cholesterol and DiPhyPC/DPPC/cholesterol mixture was kept at 55 °C and 60 °C, respectively. The GUV suspension in 150 mM sucrose solution was then slowly cooled to room temperature in a period of 8–10 h. It has been shown that non-equilibrium phase behavior can result if GUVs are cooled too quickly [22].

2.3. Preparation of GUVs by electroformation from damp lipid film

In this modified procedure, we avoid the dry lipid film as an intermediate step in order to reduce lipid demixing. We first prepare liposomes using the recently updated Rapid Solvent Exchange (RSE) method [19], then use the RSE liposomes to produce damp lipid films, and finally use the electroformation method to produce GUVs from the damp lipid films. It has been shown that liposomes made by the RSE method are homogeneous in lipid composition and free of demixing artifacts [20]. 300 μM RSE liposomes in water were prepared using the updated RSE procedure. First, lipids were dissolved in 70 μl of chloroform. The lipid solution was then heated to 50 °C briefly in a glass tube and 1.3 ml of water was added. While keeping the mixture vigorously vortexed in the glass tube, the bulk solvent was removed by gradually reducing the pressure to about 3 cm of Hg using a home-built vacuum attachment. The remaining trace chloroform was removed by an additional minute of vortexing at this same pressure [19]. The liposomes prepared by the above procedures were then sealed under argon. 20 μl of the RSE liposome suspension in water was spread on ITO coated slides to cover an area about 1 cm in diameter. After that, in order to test the effect of drying process, two different drying methods were used: (1) with the first method, the RSE liposomes on ITO coated slides were dried in room humidity (about 40%) for about 4 h, and the lipid spots looked like translucent film with some whitish spots after drying; (2) with the second method, the slides were placed inside a sealed constant humidity chamber for 22–25 h (see Fig. 2). Wet potassium carbonate (K_2CO_3) was used to keep the humidity of the chamber at 55%. The resulting damp lipid films on ITO coated slides appeared smooth and translucent. After drying, GUVs were then produced using the same electroformation procedure described above.

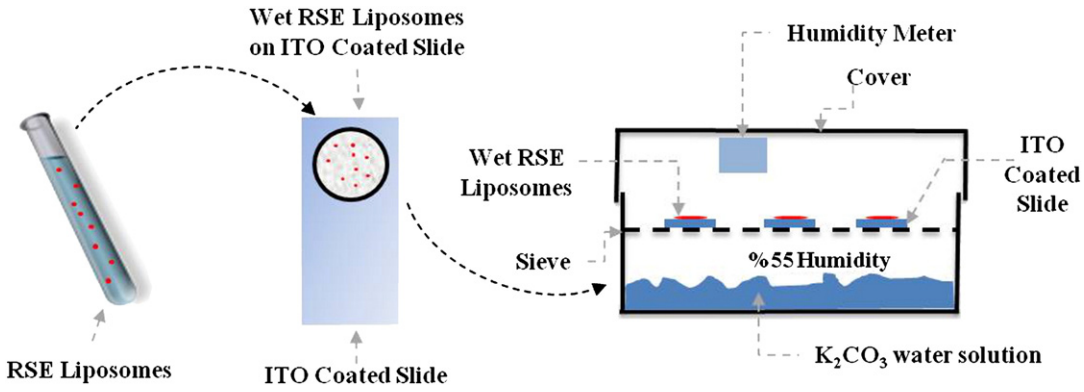

Fig. 2. The damp lipid film method.

2.4. GUV miscibility transition temperature measurement

Fluorescence images of GUVs and Nomarski differential interference contrast (DIC) image of dry lipid films were captured using a Cooke SensiCam CCD camera (Auburn Hills, MI) on an Olympus IX70 inverted microscope (Melville, NY) with either a 40× or a 20× Olympus objective. The samples were placed on a home-built temperature controlled sample stage made of copper and aluminum, with sample in direct contact with the copper part. Heating and cooling of samples were achieved by two thermoelectric Peltier modules (06311-5L31-02CFL, Custom Thermoelectric, Bishopville, MD) controlled by a thermoelectric temperature controller and a10 kΩ thermistor (WTC3293-14001-A and TCS10K5, Wavelength Electronics, Bozeman, MT). The thermistor was embedded inside the copper sample stage less than 1 mm from the sample. To ensure good thermal contact, a thin layer of thermal compound was used to attach microscope slide to the sample stage.

GUVs with bulged domains or with small vesicles attached were not selected for measurement. The uniformity of lipid composition of GUVs is judged based on the uniformity of transition temperature measured on 50 GUVs from 5 independent preparations. The selected GUVs had circular lipid domains at room temperature and were heated until domains disappeared. In order to measure the transition temperature, those homogeneous GUVs were slowly cooled down 0.5 °C at a time, and allowed to reach equilibrium at each temperature step (3–5 min/step). This process was continued until tiny dark domains appeared and the temperature was recorded as the miscibility transition temperature.

2.5. Measuring cholesterol maximum solubility by light scattering

Ninety-degree light scattering was measured using a T-mode PTI (Lawrenceville, NJ) C61/2000 spectrofluorimeter. The incident beam was set at 550 nm with a 2 nm slit width. To avoid detector saturation, the 90° scattering light was collected with the detection monochromators set at 4 nm higher than the incident wavelength. The diameter of the illumination beam in the cuvette was narrowed down to about 2 mm by adjusting the focus lens. 2 ml of 100 μM diPhyPC/cholesterol suspension in water was added to a cuvette containing a Teflon coated magnetic stir bar. Determination of cholesterol solubility by light scattering exploits the differences in size, structure and refractive index between cholesterol crystals and bilayer vesicles. As cholesterol crystals enter or leave the narrow illumination beam due to stirring motion, fluctuations were introduced in the scattering intensity. The scattering signals from both detector channels were collected in the photon counting mode at a rate of 10 data points/second for a total of 60 s.

3. Results and discussion

3.1. The issue of light-induced domains

It has been reported that exposure to light for a long period of time can cause photo oxidation of fluorescence dyes, and this may lead to artifactual domain formation (i.e., light-induced domains) [1,22,23]. The severity of the problem depends on a number of factors, including the nature of lipid mixture, the intensity of light, the type and the concentration of fluorescence molecule, and the total exposure time. In order to reduce unnecessary light exposure, we adjusted the microscope illumination diaphragm to reduce the area of illumination, so that GUVs outside of the small viewing area would not be illuminated. Also, a light shutter was used to cut illumination off whenever it was not necessary to monitor the fluorescence image continuously. We also directly measured the time for light-induced domains to occur under our experimental condition. We prepared GUVs with a lipid composition just outside the two-phase region (i.e., 28:28:46 DOPC/DSPC/cholesterol), and placed the GUVs in full illumination and waited for light-induced domains to appear. We found that it took about 15 and 25 min for light-induced domains to appear, for GUVs labeled with Rhodamine-PE and $DiI_{12}(3)$, respectively. For GUVs with a lipid composition further away from the phase boundary, the time should be even longer [22]. The cumulative exposure time for us to measure the miscibility transition temperature of an individual GUV is shorter (about 7 min). Furthermore, for some GUVs, we measured the transition temperature twice on the same GUV and found that the two measured transition temperatures were essentially the same. Thus, we concluded that under our experimental condition, our transition temperature measurements were not affected by the light-induced domain problem. Fig. 3 shows some images of a GUV from our experiment.

3.2. Distribution of transition temperature

GUVs of two ternary lipid mixtures (i.e., 1:1:1 DOPC/DSPC/cholesterol and 3:3:4 diPhyPC/DPPC/cholesterol) were produced by the electroformation method from dry lipid films as well as from damp lipid films. For each preparation method, 5 independent batches of GUVs were prepared, and 10 GUVs from each batch were selected for the transition temperature measurement. For each ternary mixture and preparation method, the average transition temperature and its standard deviation were calculated. As shown in Table 1 and Fig. 4, GUVs prepared from dry lipid film had wider distributions of transition temperature: The standard deviations were roughly ~2 °C for 1:1:1 DOPC/DSPC/cholesterol mixture and ~4 °C for 3:3:4 diPhyPC/DPPC/cholesterol mixture. The type of solvent that was used to dissolve lipids, chloroform or 2:1 chloroform/methanol, did not make a significant difference to the standard deviation.

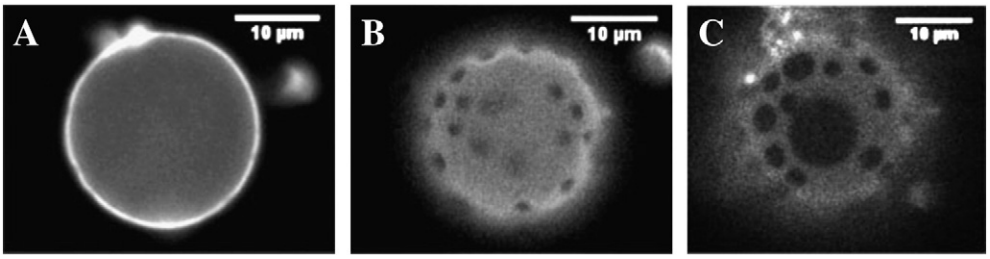

Fig. 3. Rhodamine-PE fluorescence images of a 1:1:1 DOPC/DSPC/cholesterol GUV prepared from damp lipid film. (A) At 38 °C, the GUV appeared homogeneous. (B) As temperature decreased to 34.5 °C, which is about 1 °C below the transition temperature, dark (i.e., liquid-ordered) lipid domains became visible. (C) After staying at 34.5 °C for 1 h, the dark domains became larger through coalescing.

On the other hand, GUVs prepared from wet lipid films using the 55% humidity chamber had standard deviations of *Tc* roughly 2.5 times smaller than those of GUVs prepared from dry lipid films. A smaller standard deviation in miscibility transition temperature should correspond to a more uniform distribution in GUV lipid composition. The transition temperatures and standard deviations we measured for GUVs prepared from dry lipid film were in line with those reported by other groups. For example, it has been reported that the standard deviations of measured miscibility transition temperature for diPhyPC/DPPC/cholesterol mixtures ranged 2 to 10 °C [7]. Interestingly, GUVs made from RSE liposomes dried in room humidity, which is on average around 40%, also had large standard deviations in transition temperature, only slightly smaller than that of dry film. Our data indicate that if lipid films become sufficiently dry, independent of prior history, demixing of lipids occurs, which results in a wider distribution in GUV lipid composition. However, if the drying process takes place in a higher humidity environment, such as 55%, lipid films do not dry completely and the demixing of lipids is significantly reduced. In order to have good quality damp lipid films, the drying process needs to take place slowly. We tried to place a mini-fan inside the humidity chamber to speed up the process; however, the drying became very uneven. We also tested different types of salt for the chamber, including sodium chloride (75% humidity) and potassium chloride (84% humidity). However, using these salts as constant humidity sources increased the drying time enormously (>48 h) and the majority of GUVs also had small vesicles attached.

3.3. Heterogeneity in lipid composition

It is more meaningful to estimate the lipid compositional heterogeneity of GUVs. Veatch et al. [24] constructed a temperature-composition phase diagram of DOPC/DPPC-d_{62}/cholesterol mixture with temperature as the 4th axis. That phase diagram shows that the transition temperature decreases as cholesterol content increases, and the transition temperature is not sensitive to the ratio of DOPC to DPPC in the middle of the 2-phase region. Since the phase diagrams of DOPC/DSPC/cholesterol and DOPC/DPPC-d_{62}/cholesterol have similar shape, we expect that the primary cause of heterogeneity in transition temperature of 1:1:1 DOPC/DSPC/cholesterol GUVs is the variation in cholesterol content. On the other hand, the temperature-composition phase diagram of diPhyPC/DPPC/cholesterol by Veatch et al. shows that the transition temperature is sensitive to both PC and cholesterol contents [7]. While experimental evidence of demixing of PCs in GUVs is still lacking, X-ray diffraction and optical microscopy both provided clear evidences of formation of cholesterol crystals in dry lipid films (see Section 3.5). Here, we make an attempt to estimate cholesterol compositional heterogeneity in GUVs with the assumption that the wide distributions of GUV transition temperature are primarily caused by cholesterol compositional heterogeneity. In order to translate the measured distribution in transition temperature into heterogeneity in GUV lipid composition, the transition temperatures of two additional ternary lipid mixtures, 32.5:32.5:35 DOPC/DSPC/cholesterol and 29:29:42 diPhyPC/DPPC/cholesterol, were measured for the calibration purpose; each of these mixtures contains 2 mol% more of cholesterol than their counterparts. As shown in Table 1, the average transition temperature of a 32.5:32.5:35 DOPC/DSPC/cholesterol mixture is 1.0 °C lower than that of a 1:1:1 DOPC/DSPC/cholesterol mixture, and the average transition temperature of a 29:29:42 diPhyPC/DPPC/cholesterol mixture is 0.6 °C lower than that of a 3:3:4 diPhyPC/DPPC/cholesterol mixture. Based on these results, we estimate that 1 °C variation in transition temperature in DOPC/DSPC/cholesterol mixture is roughly equivalent to 2 mol% variations in cholesterol content, and 0.6 degree variation in transition temperature of diPhyPC/DPPC/cholesterol mixture is equivalent to 2 mol% variation in cholesterol content. Thus, the standard deviation in cholesterol content

Table 1
Average miscibility transition temperatures (*Tc*) and the standard deviations for DOPC/DSPC/cholesterol and diPhyPC/DPPC/cholesterol GUVs.

Lipid mixture		# of GUV	Average *Tc* (°C)	Standard deviation (°C)
DOPC/ DSPC/Chol 1:1:1	Dry film (chloroform/methanol)	50	37.6	2.24
	Dry film (chloroform)	50	37	2.09
	Liposome dried in room humidity (30–40%)	50	36	1.57
	Damp film constant humidity: 55%	50	35.5	0.86
DOPC/DSPC/Chol 32.5:32.5:35	Damp film constant humidity: 55%	30	34.5	0.92
diPhyPC/DPPC/Chol 3:3:4	Dry film (chloroform/methanol)	50	36.9	3.97
	Dry film (chloroform)	50	36.3	3.97
	Liposome dried in room humidity (30–40%)	50	36.2	3.9
	Damp film constant humidity: 55%	50	35.9	1.62
diPhyPC/DPPC/Chol 29:29:42	Damp film constant humidity: 55%	30	35.32	1.56

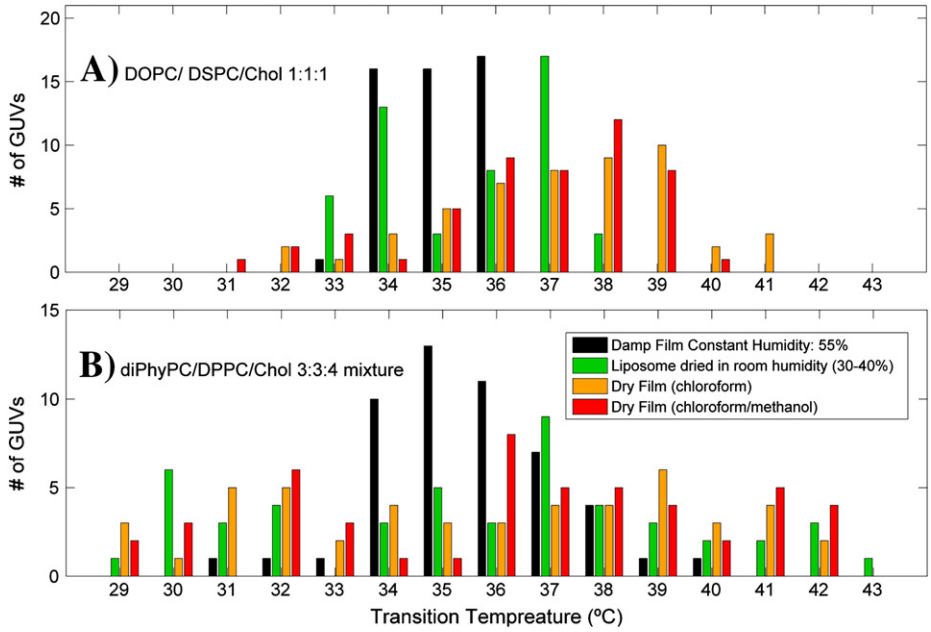

Fig. 4. Distributions of transition temperature of GUVs prepared by different methods. For each lipid mixture and preparation method, 50 GUVs from 5 independent preparations were measured.

for 1:1:1 DOPC/DSPC/cholesterol GUVs made from a dry film is estimated to be ~4 mol%, and for 3:3:4 diPhyPC/DPPC/cholesterol GUVs made from a dry film is about ~13 mol%. Thus, the variation in GUV lipid composition is quite significant. The damp lipid film method developed in this study can effectively reduce lipid composition heterogeneity and improve the accuracy of measurements.

3.4. Lipid demixing and cholesterol maximum solubility

Although the same experimental procedure was used for both mixtures, the standard deviation in lipid composition is larger for diPhyPC/DPPC/cholesterol GUVs prepared from dry lipid films. A number of factors could contribute to the difference, such as the locations of mixtures in the corresponding phase diagrams and the budding tendencies of lipid domains [2,7,25]. Another likely factor is the unfavorable interaction between diPhyPC and cholesterol, which is reflected by the low solubility limit of cholesterol in diPhyPC bilayers. Previously, it has been shown that the demixing of lipids in dry state becomes worse in a lipid mixture with cholesterol mole fraction close to the cholesterol maximum solubility limit in that bilayer [26]. Using X-ray diffraction, light scattering, and a cholesterol oxidase (COD) assay, the maximum solubility of cholesterol in many phosphatidylcholine bilayers, including DOPC, POPC, DSPC, and DPPC, was found to be 66 mol% [26–28]. In this study, we measured the maximum solubility of cholesterol in diPhyPC lipid bilayers using light scattering. Previously, it had been shown that the standard deviation of scattering intensity becomes significantly larger when cholesterol mole fraction of a lipid mixture is beyond the solubility limit of cholesterol, due to the scattering of light by cholesterol monohydrate crystals [28]. In Fig. 5, the standard deviation of light scattering intensity normalized by the average scattering intensity is plotted as a function of cholesterol mole fraction for diPhyPC/cholesterol binary mixtures. Clearly, the sharp increase occurs around ~40 mol% of cholesterol. The maximum solubility of cholesterol in diPhyPC lipid bilayers determined from three independent experiments was 40.2 ± 0.5 mol%, which is far lower than that in other PC lipid bilayers. A recent study of cholesterol maximum solubility in POPE/POPC mixtures showed that the maximum solubility increases linearly with the ratio of POPC/(POPC + POPE), from 50 mol% for pure POPE to 66 mol% for pure POPC [29]. Assuming that the maximum solubility of cholesterol in diPhyPC/DPPC mixtures also increases linearly with the ratio of DPPC/(DPPC + diPhyPC), the expected solubility value in 1:1 diPhyPC/DPPC mixture will be 52 mol%, which is significantly lower than that in DOPC/DSPC mixtures (66 mol%). The low cholesterol solubility in diPhyPC facilitates the demixing of lipid components in dry state, which results in a higher heterogeneity in lipid composition for diPhyPC/DPPC/cholesterol GUVs.

Previously, we proposed the Umbrella Model to explain the key molecular interaction between cholesterol and phospholipids [27,30]. Cholesterol has a large nonpolar steroid ring body and a relatively small polar hydroxyl headgroup. When cholesterols are incorporated into a phospholipid bilayer, neighboring phospholipid headgroups provide cover to shield the nonpolar part of cholesterol from exposure to water in order to avoid the unfavorable free energy. Thus, phospholipid headgroups act like umbrellas and the space under the headgroups is shared by acyl chains and cholesterols.

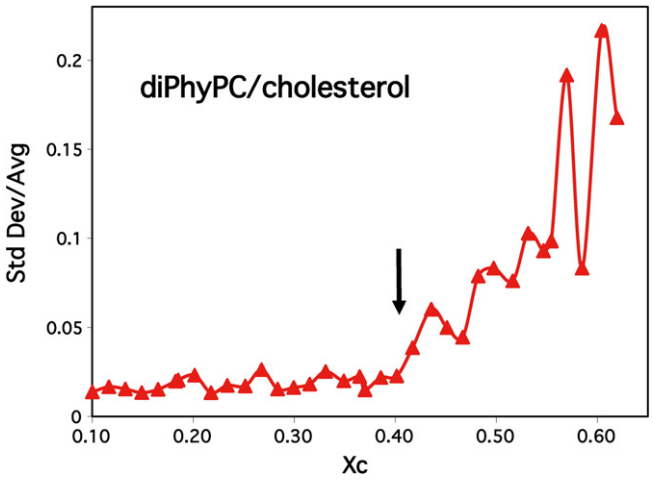

Fig. 5. Light scattering detects cholesterol precipitation from bilayers. Standard deviation of light scattering intensity normalized by the average intensity as a function of cholesterol mole fraction in diPhyPC/cholesterol mixtures. The sharp rise near cholesterol mole fraction of 0.4 results from the scattering of light by cholesterol monohydrate crystals.

With the Umbrella Model, the maximum solubility limit of cholesterol in a lipid bilayer is interpreted as the cholesterol mole fraction at which the capability of phospholipid headgroups to cover cholesterol molecules from water has reached its maximum and any additional cholesterol would precipitate from the lipid bilayer and form cholesterol monohydrate crystals. DiPhyPC is a very unusual phosphatidylcholine: its acyl chains are bulky, because of the four additional methyl groups on each of its chains (see Fig. 1). Thus, there is much less space under diPhyPC headgroups for cholesterol and interactions between two are quite unfavorable, compared to other PCs.

3.5. Lipid demixing occurs in the dry film state

Our results show that for GUVs prepared from dry lipid films, the solvent used (chloroform or 2:1 chloroform/methanol) to dissolve lipids did not make a noticeable difference in terms of composition heterogeneity of GUV. This is understandable, since solvent was all evaporated in the dry film state. Even with RSE liposomes, which are known to have good uniformity in lipid composition, the result was similar to dry film, if the liposomes become sufficient dry (see Table 1 and Fig. 4). These results strongly indicate that lipid demixing occurs in the dry film state.

In a previous X-ray diffraction study, it was found that cholesterol molecules demix from phospholipids in dry lipid films, and form anhydrous cholesterol crystals [26]. Once cholesterol crystals are formed in the dry state, not all crystals will be dissolved even after months of hydration [26]. We tried to visualize cholesterol crystals in dry lipid films using optical microscopy. We observed micron-sized crystals in some dry lipid films containing cholesterol, and the sizes of crystals grow with time. The general pattern is that the higher the cholesterol content, the more the crystals; the lower the cholesterol content, the longer the incubation period required. For example, large quantity of crystals was seen in dry lipid film of 15:15:70 DOPC/DSPC/cholesterol mixture, as soon as the sample was prepared. For dry film of 4:6 DOPC/cholesterol, crystals can only be observed after 3 days of incubation at room temperature (Fig. 6D). After 2 weeks of incubation, crystals were observed in the dry film of 25:25:50 DOPC/DSPC/cholesterol mixture (Fig. 6B), but not in 3:3:4 DOPC/DSPC/cholesterol mixture. Interestingly, crystals were detected in the dry film of 3:3:4 diPhyPC/DPPC/cholesterol mixture (Fig. 6C) after two weeks of incubation. This result is significant, because the same mixture was used for the GUV transition temperature measurement (Table 1). Combining these results with the result from previous X-ray diffraction experiment, those observed crystals should be anhydrous cholesterol crystals. It should be pointed out that unlike X-ray diffraction, optical microscopy couldn't detect crystals if the size of the crystals is below the resolution limit of optical microscopy. Thus, even if optical microscopy detects no crystal, we cannot conclude that there is not crystal in the sample. In any case, the observation of cholesterol crystals in some dry lipid films supports our assessment that cholesterol demix from other lipids in dry state. In addition, since micron-sized cholesterol crystals were observed in 3:3:4 diPhyPC/DPPC/cholesterol dry films, but not in 1:1:1 DOPC/DSPC/cholesterol dry film after 2 weeks incubation, demixing of lipids in dry state is more severe for 3:3:4 diPhyPC/DPPC/cholesterol mixture.

Another indication of cholesterol demixing can be found in the values of transition temperature. Since some cholesterol will stay as crystals even after months of hydration [26], the average cholesterol mole fraction of GUV will be less than the cholesterol mole fraction of the sample, which should result in a higher average miscibility transition temperature for GUVs. A careful inspection of Table 1 and Fig. 4 shows that this is indeed the case: for both ternary mixtures, the average GUV transition temperature is the highest for dry film preparations and the lowest for damp film preparations. In order to judge whether the differences in average transition temperature for dry and damp film preparations are statistically meaningful, we

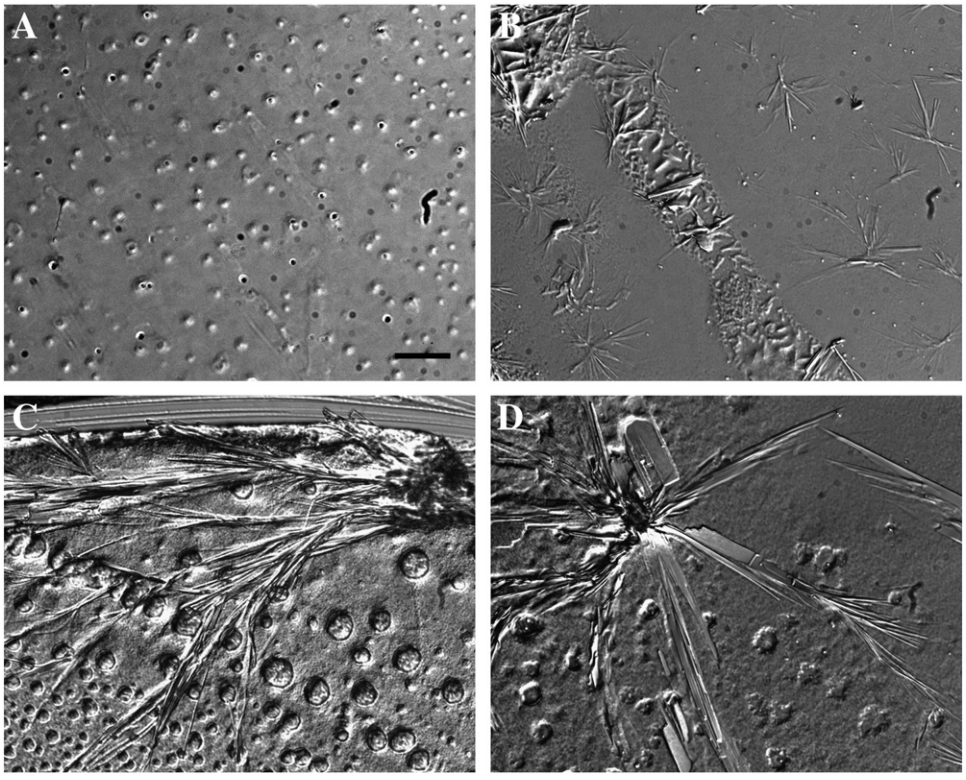

Fig. 6. DIC images show cholesterol demixing from other lipids and forming cholesterol crystals in dry lipid films. The length of the black bar is 50 μm. (A) 2-week-old dry lipid film of DOPC, no crystal. (B) 2-week-old dry film of 25:25:50 DOPC/DSPC/cholesterol. (C) 2-week-old dry film of 3:3:4 diPhyPC/DPPC/cholesterol. (D) 3-day-old dry film of 4:6 DOPC/cholesterol.

performed Student's *t*-test using the values in Table 1. For 1:1:1 DOPC/DSPC/cholesterol mixture, the *t* value, degrees of freedom, and two-tailed test *p*-value are 6.19, 63 and 0.000, respectively. Thus, the difference in transition temperature is definitely significant. For 3:3:4 diPhyPC/DPPC/cholesterol mixture, the calculated values are 1.65, 65 and 0.052. Thus, the difference may or may not be significant due to large standard deviation of the data. Our results indicate that GUVs prepared by the damp film method contain more cholesterol than GUVs prepared by other methods. Thus, the demixing of lipids not only increases the standard deviation of GUV lipid composition, but also lowers the average cholesterol mole fraction of GUVs.

3.6. Potential of the damp lipid film method

With the damp lipid film method, wet liposomes are transformed into GUVs without going through a dry state. Thus, the method has a potential to directly prepare GUVs from proteo-liposomes or cell membranes containing native membrane proteins. Also, we have been successful in making GUVs with the damp film method in a buffer containing up to 50 mM KCl (5 mM PIPES, 50 mM KCl, 1 mM EDTA, 1 mM NaN_3, pH 7.0) following the electroformation method of Pott and Bouvrais [15]. Preparing GUVs in ionic buffer is of course more biologically relevant.

4. Conclusions

In this work, a new procedure to produce GUVs with better uniformity in lipid composition has been developed. Lipid compositional uniformity was measured through the distribution of GUV miscibility transition temperatures. This work shows that the deviation in GUV lipid composition prepared from a dry lipid film is high and a major cause is the demixing of lipid components in the dry film state. The problem becomes worse for a lipid mixture in which cholesterol has a low maximum solubility limit.

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References

[1] S.L. Veatch, S.L. Keller, Separation of liquid phases in giant vesicles of ternary mixtures of phospholipids and cholesterol, Biophys. J. 85 (2003) 3074–3083.
[2] J. Zhao, J. Wu, F.A. Heberle, T.T. Mills, P. Klawitter, G. Huang, G. Costanza, G.W. Feigenson, Phase studies of model biomembranes: complex behavior of DSPC/DOPC/cholesterol, Biochim. Biophys. Acta 1768 (2007) 2764–2776.
[3] N. Kahya, Protein–protein and protein–lipid interactions in domain-assembly: lessons from giant unilamellar vesicles, Biochim. Biophys. Acta-Biomembr. 1798 (2010) 1392–1398.
[4] K. Carvalho, L. Ramos, C. Roy, C. Picart, Giant unilamellar vesicles containing phosphatidylinositol(4,5) bisphosphate: characterization and functionality, Biophys. J. 95 (2008) 4348–4360.
[5] P. Streicher, P. Nassoy, M. Baermann, A. Dif, V. Marchi-Artzner, F. Brochard-Wyart, J. Spatz, P. Bassereau, Integrin reconstituted in GUVs: a biomimetic system to study initial steps of cell spreading, Biochim. Biophys. Acta-Biomembr. 1788 (2009) 2291–2300.
[6] A.R. Honerkamp-Smith, S.L. Veatch, S.L. Keller, An introduction to critical points for biophysicists; observations of compositional heterogeneity in lipid membranes, Biochim. Biophys. Acta-Biomembr. 1788 (2009) 53–63.
[7] S.L. Veatch, K. Gawrisch, S.L. Keller, Closed-loop miscibility gap and quantitative tie-lines in ternary membranes containing diphytanoyl PC, Biophys. J. 90 (2006) 4428–4436.
[8] C. Esposito, A. Tian, S. Melamed, C. Johnson, S.-Y. Tee, T. Baumgart, Flicker spectroscopy of thermal lipid bilayer domain boundary fluctuations, Biophys. J. 93 (2007) 3169–3181.
[9] A.R. Honerkamp-Smith, P. Cicuta, M.D. Collins, S.L. Veatch, M. den Nijs, M. Schick, S.L. Keller, Line tensions, correlation lengths, and critical exponents in lipid membranes near critical points, Biophys. J. 95 (2008) 236–246.
[10] S.L. Veatch, S.L. Keller, Seeing spots: complex phase behavior in simple membranes, Biochim. Biophys. Acta-Mol. Cell Res. 1746 (2005) 172–185.
[11] G.W. Feigenson, Phase behavior of lipid mixtures, Nat. Chem. Biol. 2 (2006) 560–563.
[12] D. Scherfeld, N. Kahya, P. Schwille, Lipid dynamics and domain formation in model membranes composed of ternary mixtures of unsaturated and saturated phosphatidylcholines and cholesterol, Biophys. J. 85 (2003) 3758–3768.
[13] T. Baumgart, G. Hunt, E.R. Farkas, W.W. Webb, G.W. Feigenson, Fluorescence probe partitioning between L-o/L-d phases in lipid membranes, Biochim. Biophys. Acta-Biomembr. 1768 (2007) 2182–2194.
[14] M. Angelova, S. Soléau, P. Méléard, F. Faucon, P. Bothorel, C. Helm, M. Lösche, H. Möhwald, Preparation of giant vesicles by external AC electric fields, Kinetics and Applications Trends in Colloid and Interface Science VI, vol. 89, Springer Berlin, Heidelberg, 1992, pp. 127–131.
[15] T. Pott, H. Bouvrais, P. Méléard, Giant unilamellar vesicle formation under physiologically relevant conditions, Chem. Phys. Lipids 154 (2008) 115–119.
[16] K. Akashi, H. Miyata, H. Itoh, K. Kinosita, Preparation of giant liposomes in physiological conditions and their characterization under an optical microscope, Biophys. J. 71 (1996) 3242–3250.
[17] L.R. Montes, A. Alonso, F.M. Goni, L.A. Bagatolli, Giant unilamellar vesicles electroformed from native membranes and organic lipid mixtures under physiological conditions, Biophys. J. 93 (2007) 3548–3554.
[18] C.C. Vequi-Suplicy, K.A. Riske, R.L. Knorr, R. Dimova, Vesicles with charged domains, Biochim. Biophys. Acta-Biomembr. 1798 (2010) 1338–1347.
[19] J.T. Buboltz, A more efficient device for preparing model-membrane liposomes by the rapid solvent exchange method, Rev. Sci. Instrum. 80 (2009) 124301.
[20] J.T. Buboltz, G.W. Feigenson, A novel strategy for the preparation of liposomes: rapid solvent exchange, Biochim. Biophys. Acta-Biomembr. 1417 (1999) 232–245.
[21] P.B. Kingsley, G.W. Feigenson, The synthesis of a perdeuterated phospholipid: 1,2-dimyristoyl-sn-glycero-3-phosphocholine-d72, Chem. Phys. Lipids 24 (1979) 135–147.
[22] N.F. Morales-Penningston, J. Wu, E.R. Farkas, S.L. Goh, T.M. Konyakhina, J.Y. Zheng, W.W. Webb, G.W. Feigenson, GUV preparation and imaging: minimizing artifacts, Biochim. Biophys. Acta-Biomembr. 1798 (2010) 1324–1332.
[23] A.G. Ayuyan, F.S. Cohen, Lipid peroxides promote large rafts: effects of excitation of probes in fluorescence microscopy and electrochemical reactions during vesicle formation, Biophys. J. 91 (2006) 2172–2183.
[24] S.L. Veatch, O. Soubias, S.L. Keller, K. Gawrisch, Critical fluctuations in domain-forming lipid mixtures, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 17650–17655.
[25] A. Tian, B.R. Capraro, C. Esposito, T. Baumgart, Bending stiffness depends on curvature of ternary lipid mixture tubular membranes, Biophys. J. 97 (2009) 1636–1646.
[26] J.Y. Huang, J.T. Buboltz, G.W. Feigenson, Maximum solubility of cholesterol in phosphatidylcholine and phosphatidylethanolamine bilayers, Biochim. Biophys. Acta-Biomembr. 1417 (1999) 89–100.
[27] J. Huang, Model membrane thermodynamics and lateral distribution of cholesterol: from experimental data to Monte Carlo simulation, Methods Enzymol. 455 (2009) 329–364.
[28] A. Parker, K. Miles, K.H. Cheng, J. Huang, Lateral distribution of cholesterol in dioleoylphosphatidylcholine lipid bilayers: cholesterol–phospholipid interactions at high cholesterol limit, Biophys. J. 86 (2004) 1532–1544.
[29] S. Balyimez, S. Park, J. Huang, The maximum solubility of cholesterol in POPC/POPE lipid mixtures, Biophys. J. 100 (2011) 625a.
[30] J.Y. Huang, G.W. Feigenson, A microscopic interaction model of maximum solubility of cholesterol in lipid bilayers, Biophys. J. 76 (1999) 2142–2157.